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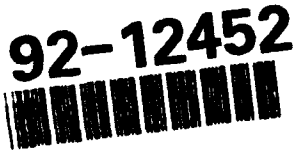

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The purpose of this study was to determine the involvement of cyclooxygenase and lipoxygenase metabolites in the depression of host defense induced by thermal injury in a guinea pig model. We found that the bactericidal defect of neutrophils induced by thermal injury in this model was related to a marked elevation of intracellular cyclic-3',5'-adenosine monosphate (cAMP), which in turn was related to autogenous prostaglandin (PG) E_1 production by these cells. Suppression of PGE_1 production by in vivo or in vitro treatment of neutrophils with selective cyclooxygenase inhibitors normalized intracellular cAMP and correct the bactericidal defect. Despite the profound effects of cyclooxygenase inhibitors on neutrophil function, these drugs were not found to influence the depression of cell-mediated immune responses induced by thermal injury in guinea pig or

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mouse models. These drugs also had no effect on survival or the time to death following experimental burn wound sepsis with *Pseudomonas aeruginosa* or *Proteus mirabilis*. No evidence was obtained supporting involvement of lipoxygenase metabolites in the bactericidal defect of neutrophils or the depression of cell-mediated immune responses induced by thermal injury. In summary, our study has pinpointed the mechanism responsible for the bactericidal defect of neutrophils in a guinea pig model of thermal injury. In addition, our results ruled out a major role for cyclooxygenase or lipoxygenase metabolites in mediating the depression of cell-mediated immune responses following thermal injury in several animal models.

FOREWORD

In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985). Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF FIGURES AND TABLES

BODY OF REPORT

Statement of the Problem Under Study

Sepsis remains a leading cause of morbidity and mortality in patients who have sustained thermal injury [1]. The increased incidence of infection in these patients is related to loss of the protective skin barrier and to the profound depression of host defense mechanisms induced by severe injury [reviewed in 2]. The advent of topical antimicrobial therapy, the development of more effective topical and systemic antibiotics, and early excision and grafting of the burn wound have decreased the mortality directly related to bacterial infection in thermally injured patients [1]. Further progress in reducing mortality due to infection in these patients is dependent on understanding the fundamental mechanisms responsible for the depression of host defense induced by thermal injury.

Background and Rationale

Studies reported to date on alterations of host defense induced by thermal injury have been primarily descriptive in nature. These studies have produced a wealth of information documenting the alterations in the inflammatory and immune systems that occur following thermal injury [reviewed in 2]. These alterations include depression of levels of circulating immunoglobulins, complement and fibronectin; reduction in serum opsonic activity; dysfunction of neutrophils and monocytes; decrease in cell-mediated immune responses; increase in metabolism of arachidonic acid; depression of clearance of particulate material by the reticuloendothelial system; and appearance of circulating factors that reduce the functions of phagocytic cells and lymphocytes. However, studies in this area have failed to identify the mechanisms underlying these alterations. This

information is essential for the development of rational therapeutic approaches for reversing or circumventing host defense alterations during recovery from thermal injury.

Our early studies in this area defined various humoral alterations of host defense in thermally injured patients (i.e., serum opsonic deficiency, various alterations of complement and serum-mediated inhibition of neutrophil function) [3-10]. These studies determined the relationship among the various humoral alterations and correlations with the extent of thermal injury and the occurrence of septic episodes. Our subsequent studies have been directed towards determining the mechanisms responsible for various cellular alterations of host defense induced by thermal injury in an animal model. A guinea pig model was selected for our studies for three major reasons. First, the metabolic response to thermal injury in guinea pigs has been shown to simulate the response occurring in humans [11]. Second, hematologic changes in the guinea pig model have been shown to resemble those observed in thermally injured humans, except for the lack of leukopenia which is probably compensated for by splenic hematopoiesis in the guinea pig and other rodents [12]. Third, unlike rats and mice, guinea pigs are "corticosteroid resistant" like humans, and therefore immunologic changes associated with endogenous glucocorticoid production should be similar in these species [13].

Our first studies with the guinea pig model documented the presence of immunologic and hematologic alterations resembling those occurring in thermally injured humans [14]. The following immunologic alterations were shown to occur early after injury and resolved by the end of the first week postburn: depression of the inherent bactericidal activity of neutrophils against *Pseudomonas aeruginosa*, appearance of serum factors that inhibited the bactericidal activity of normal neutrophils, reduction in bacterial

clearance by the reticuloendothelial system, systemic complement consumption and increased generation of arachidonate metabolites of the cyclooxygenase pathway. In contrast, depression of lymphoproliferative responses to T cell mitogens was maximal at four days postburn and resolved by three weeks postburn [14]. These results suggested that there was a continuum of alterations in the inflammatory and immune systems resulting from thermal injury and that alterations in the inflammatory system were a direct consequence of injury.

The observed temporal association between production of arachidonate metabolites of the cyclooxygenase pathway and depression of the bactericidal activity of neutrophils suggested that cyclooxygenase metabolites might be involved in mediating neutrophil dysfunction induced by thermal injury. As a first step in testing this hypothesis, we began the investigation summarized in this document by determining the effects of classic cyclooxygenase inhibitors on the bactericidal activity of neutrophils in the guinea pig model. We also determined the effects of the cyclooxygenase inhibitors on other key immunologic and hematologic alterations in this model.

Effects of Cyclooxygenase Inhibitors on Immunologic and Hematologic Alterations Induced by Thermal Injury

The effects of parenteral therapy with indomethacin, ibuprofen and piroxicam on the bactericidal activity of peripheral neutrophils against *P. aeruginosa*, serum complement, proliferative responses of splenic lymphocytes to T cell mitogens, complete blood count and platelet count were determined in the guinea pig model. Therapy with the nonsteroidal anti-inflammatory drugs (NSAIDs) was initiated at 3 h postburn to simulate conditions that could be potentially applied to the clinical care of thermally

injured patients. NSAIDs then were administered daily for a period of nine days postburn. Preliminary studies confirmed that the doses of NSAIDs used in our study (10-20 mg/kg) effectively inhibited prostaglandin and thromboxane production in wound fluid obtained from the injured area, the major site of synthesis of these compounds [15,16]. Thus, the NSAIDs acted as potent cyclooxygenase inhibitors under the conditions of our experiments.

The results of our study demonstrated that therapy with all three NSAIDs fully restored the bactericidal activity of neutrophils from the thermally injured animals to normal without having a major impact on complement, lymphoproliferative responses to T cell mitogens or the hematologic parameters measured [15,16]. These results suggested that the bactericidal defect of neutrophils induced by thermal injury in the guinea pig model was reversible and that the mechanisms responsible for this defect were inhibitable by NSAIDs. Our results also suggested that NSAIDs exerted their effect on neutrophils without involving complement. The NSAIDs may have acted by suppressing production of prostaglandins of the E series, which are known to inhibit various effector functions of neutrophils. NSAIDs also may have enhanced bactericidal activity by affecting early steps in neutrophil activation independently of cyclooxygenase inhibition. Subsequent studies described below further investigate these concepts.

Our failure to observe a major impact of NSAIDs on the depression of lymphoproliferative responses to T cell mitogens associated with thermal injury is consistent with recently published results from several other laboratories [17,18]. Although delayed type hypersensitivity and IL-2 production by splenic lymphocytes have been shown to be enhanced by NSAIDs in thermally injured animals [19,20], NSAIDs have not been found to consistently affect proliferation of lymphocytes or changes in T cell subsets induced by thermal injury [17,18]. In our studies, negative results with

NSAIDs on cell-mediated immune responses were obtained both in guinea pig and mouse models of thermal injury. The results with the mouse model are detailed in a subsequent section.

Effects of Cyclooxygenase Inhibitors on Survival from Burn Wound Sepsis

To determine if the enhancement of the bactericidal activity of neutrophils mediated by NSAIDs led to an increase in the overall resistance against bacterial infection, the effects of NSAIDs on survival following experimental burn wound sepsis with *P. aeruginosa* and *Proteus mirabilis* were investigated [16]. The bacteria were spread over the surface of the burn wound to simulate conditions that occur naturally as a result of extensive colonization of the burn wound with bacteria. Infection caused by both organisms was invasive, and death occurred in approximately fifty percent of the animals by five days post infection. NSAIDs administered at three hours postburn and then daily for nine days postburn in doses ranging from 5-20 mg/kg did not influence either survival or the time to death following infection with the test organisms. When the bacterial challenge was delayed to one day postburn to give the NSAIDs time to act before infection, again no effect on survival was observed.

Our observations suggest that the enhancement of bactericidal activity of neutrophils mediated by NSAIDs was not by itself sufficient to increase the overall resistance of the animals to lethal infection. NSAIDs may not alter the impairment in chemotaxis of neutrophils induced by thermal injury, even though these drugs correct the bactericidal defect of these cells. The increased capacity of the neutrophils to kill bacteria would be of limited value if the cells were unable to mobilize to sites of infection. Another explanation for our results is that cell-mediated immunity also may be required for defense against infection caused by these virulent

organisms, and our study failed to demonstrate an effect of NSAIDs on this aspect of host resistance. Less gross endpoints than those used in our study may provide more meaningful information in future studies concerning the effects of NSAIDs and other treatments on resistance against infection.

Mechanisms Underlying the Bactericidal Defect of Neutrophils Induced by Thermal Injury

Prostaglandins of the E series are known to inhibit various effector functions of neutrophils including chemotaxis, aggregation, superoxide production and lysosomal enzyme release [21-27]. These prostaglandins exert their effect by acting as adenylate cyclase agonists, raising intracellular cyclic-3',5'-adenosine monophosphate (cAMP) to an abnormally high level [21-24]. It has been suggested that elevation of cAMP serves as an important signal for the cell to stop functioning, replenish energy supplies and replace the surface membrane. This normal regulatory mechanism may be called into play in response to the heightened activation of neutrophils induced by thermal injury resulting in down regulation of effector functions. The next part of our study tested the hypothesis that autogenous prostaglandin E (PGE) production by neutrophils induced by thermal injury elevates intracellular cAMP, and this in turn is responsible for defective bactericidal activity of these cells.

The following evidence was obtained in the guinea pig model supporting this hypothesis [28,29]. A marked increase in cAMP content of peripheral and peritoneal exudate neutrophils was observed at one and two days post-burn in association with reduction in bactericidal activity against *P. aeruginosa*. Production of PGE_1, the major E type prostaglandin produced by neutrophils [30], was increased concomitantly. Treatment of neutrophils in vitro or in vivo with indomethacin, ibuprofen or piroxicam restored bactericidal activity to normal and concomitantly reduced cAMP content and PGE_1

production by the neutrophils. A concomitant reduction in cAMP content and PGE_1 production also was observed as bactericidal activity of neutrophils returned to normal under natural conditions during four to seven days post-burn. The enhancement of bactericidal activity of neutrophils mediated by NSAIDs was fully counteracted by purified PGE_1, theophylline and by cAMP itself.

PGE_1 has been shown to synergize with neutrophil activators to increase and maintain intracellular cAMP in neutrophils at very high levels, and this mechanism appears to be central to the marked elevation of intracellular cAMP induced by thermal injury in the guinea pig model. However, it is important to note that other mediators also may contribute to the elevation of intracellular cAMP in neutrophils including various adenylate cyclase agonists produced in response to thermal injury [31] and glucocorticoids that potentiate adenylate cyclase activation [24]. Additional mechanisms also may be involved in the elevation of intracellular cAMP in neutrophils following thermal injury.

Our study is the first to demonstrate that NSAIDs reduce the cAMP content of inflammatory neutrophils. This represents a major previously unrecognized property of these drugs. Our results suggest that NSAIDs may reduce cAMP content of inflammatory neutrophils through cyclooxygenase inhibition with suppression of PGE_1 production. However, additional mechanisms also may be involved. Concentrations of NSAIDs as high as those used in our in vitro studies have been shown to inhibit activation of normal neutrophils [32-34]. It has been proposed that NSAIDs interfere with the triggering of cellular processes independently of cyclooxygenase inhibition by blocking the interaction between G protein and the receptor for the ligand [34]. However, this mechanism would not explain our finding

that NSAIDs corrected the bactericidal defect of neutrophils from injured animals when employed in vitro, since activation of these cells had already occurred in vivo. There is little known about the effects of NSAIDs on neutrophils after they have undergone activation; our results suggest that the drugs modulate cAMP during this phase.

Involvement of PGE_2 and Interferon Gamma in the Depression of Cell-Mediated Immune Responses Induced by Thermal Injury

The mechanisms responsible for the depression of cell-mediated immune responses induced by thermal injury are largely unknown. One mechanism that appears to be involved is the induction of T suppressor cells that suppress various manifestations of T lymphocyte function [reviewed in 2]. Two mediators that are known to contribute to the induction of T suppressor cells are PGE_2 and interferon gamma (IFN_γ) [35-42]. These mediators act alone and in concert to induce T suppressor cells. Because of the recognized role of PGE_2 and IFN_γ in suppression of T lymphocyte function, we next investigated the involvement of these mediators in the depression of cell-mediated immune responses induced by thermal injury. A mouse model was used in these studies because of the recognized advantage of the mouse over other animals for assessment of lymphocyte function. Results from these and subsequent studies will be detailed, since these studies have not been presented in our previous annual reports.

C57BL/6J female mice, 10 to 12 weeks old and weighing approximately 25 grams, were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN. The mice were anesthetized by intraperitoneal injection of 65 mg/kg sodium pentobarbital followed by inhalation of methoxyflurane as needed until the animal displayed deep regular breathing. Dorsal hair was shaved, and 1 ml of lactated Ringer's solution was injected intraperitoneally. The animal was placed in a custom-made insulated mold that exposed an area on the

dorsum equal to 15% of the total body surface. The area was immersed in 99°C water for 7 seconds. The animals were given oxygen and rested for 10 to 15 min on heating blankets (set on low) to reduce heat loss and minimize stress. A second 1 ml dose of Ringer's solution was administered intraperitoneally at 1.5 h after burning. Sham injury was effected by immersing animals in tepid water. All other procedures were identical to those used with the burned animals, except oxygen was not administered.

Initial experiments were directed towards determining the time frame during which cell-mediated immune responses were depressed in the mouse model. Groups of three injured and sham-treated mice were sacrificed by CO_2 narcosis at various time intervals during three weeks postburn. Spleens were removed aseptically, and single cell suspensions were prepared. Spleen cells from injured and sham-treated mice were separately pooled. Proliferative responses of the pooled spleen cells to concanavalin A (Con A) and phytohemagglutinin (PHA) were measured as described in our previous studies, except the tissue culture medium contained 10% Nuserum V (Collaborative Research Inc., Bedford, MA) rather than fetal calf serum and 1% L-glutamine in addition to other supplements [14]. For measurement of the in vitro primary immune response to sheep erythrocytes, 1.0×10^7 pooled spleen cells in 1 ml of RPMI containing 10% Nuserum V, 1% non-essential amino acids, 1% pyruvate, 0.1% gentamicin, and 0.0004% 2-mercaptoethanol and 50 μl of 1% sheep erythrocytes (Colorado Serum, Denver, CO) were incubated in 24-well tissue culture plates for four days at 37°C in 5% CO_2. At the end of incubation period, plaque-forming cells were enumerated by the method of Cunningham and Szenberg [43]. Significant differences between data in these and subsequent experiments were determined by analysis of variance [44].

Proliferative responses of spleen cells to mitogens began to decline at 4 days postburn, were maximally reduced at 10 days postburn, and were returning to normal by 21 days postburn (Figures 1 and 2). The differences in responses between injured and sham-treated animals were significant ($P<0.05$) at 7, 10 and 14 days postburn for Con A (Figure 1) and at 4, 7, 10 and 14 days postburn for PHA (Figure 2). The in vitro primary immune response to sheep erythrocytes was significantly reduced in injured animals as compared with sham-treated animals during 2 to 21 days postburn ($P<0.05$; Figure 3). Thus, immunosuppression in this model became manifest early during the first week postburn and continued through two weeks postburn. This temporal pattern is similar to that observed in the guinea pig model [14].

We reasoned that if PGE_2 was involved in the depression of cell-mediated immune responses induced by thermal injury, then more PGE_2 should be produced by spleen cells from injured animals as compared with sham-treated animals. To test this hypothesis, we measured spontaneous production of PGE_2 by spleen cells harvested from injured and sham-treated animals at 7 days postburn, a time when cell-mediated immune responses were markedly depressed. Spleen cells (2.5×10^7 cells/ml) were incubated for 66 h at 37°C in tissue culture medium in the absence of exogenous activators. At various time intervals during the incubation period, the cells were removed by centrifugation, and prostaglandins in the supernatants were extracted as described in our previous studies [28]. PGE_2 in the extracts was measured using a radioimmunoassay kit from Advanced Magnetics (Cambridge, MA). Production of PGE_2 by spleen cells from injured animals was significantly increased as compared with spleen cells from sham-treated animals during 24 h of incubation ($P<0.05$; Figure 4). These results supported the concept that depression of cell-mediated immune responses following thermal injury might be related to increased PGE_2 production.

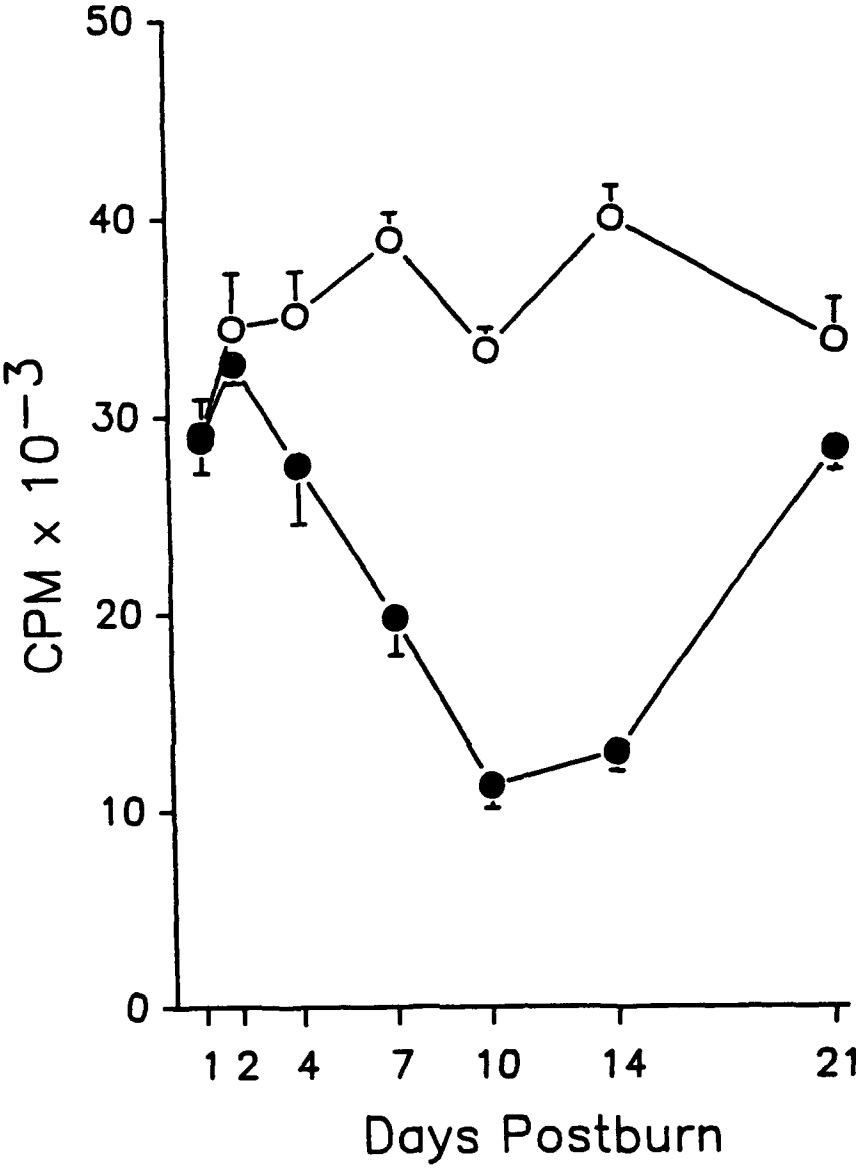

Figure 1. Proliferative responses of spleen cells to Con A during three weeks postburn. Spleen cells were harvested from three injured animals (closed circles) and three sham-treated animals (open circles) at various time intervals postburn. The cells from each group were separately pooled and then assayed. Data are mean ± SEM; n, 3.

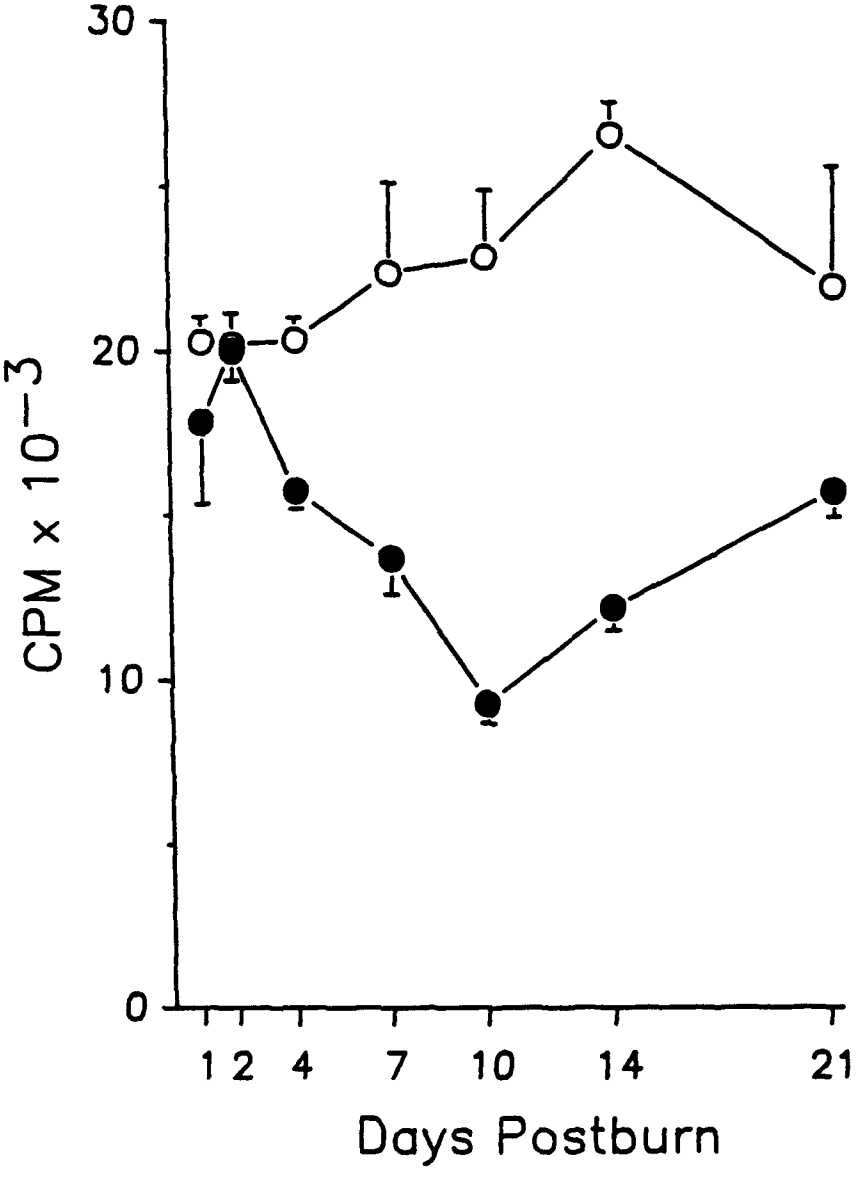

Figure 2. Proliferative responses of spleen cells to PHA during three weeks postburn. Spleen cells were harvested from three injured animals (closed circles) and three sham-treated animals (open circles). The cells from each group were separately pooled and then assayed. Data are mean ± SEM; n, 3.

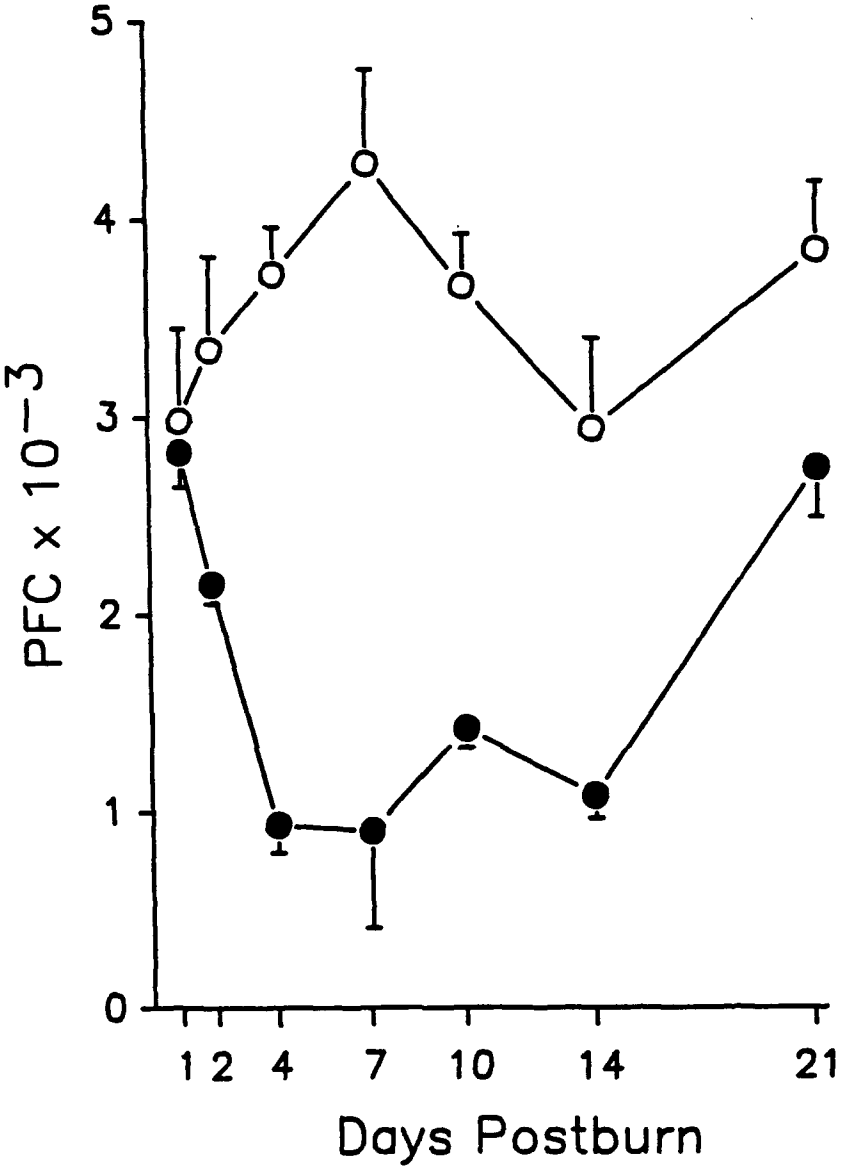

Figure 3. In vitro primary immune response of spleen cells to sheep erythrocytes during three weeks postburn. Spleen cells were harvested from three injured animals (closed circles) and three sham-treated animals (open circles) at various time intervals postburn. The cells from each group were separately pooled and then assayed. Data are mean ± SEM; n, 4.

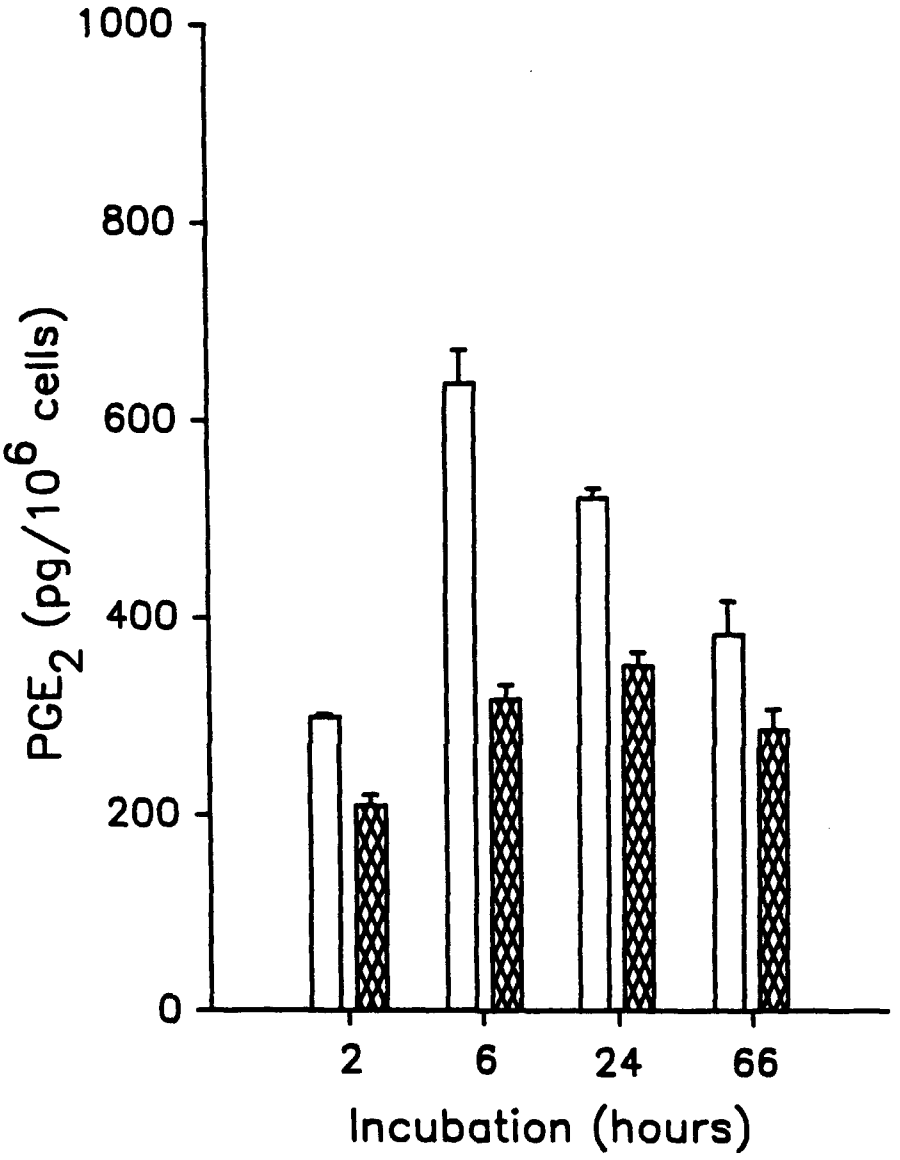

Figure 4. Autogenous PGE_2 production by spleen cells during short-term incubation in vitro. Spleen cells were harvested from injured animals (open bars) and sham-treated animals (cross-hatch bars) at seven days post-burn. The spleen cells were incubated for 66 h in vitro in the absence of activators, and PGE_2 in the supernatants was measured at various time intervals during the incubation period. Data are mean ± SEM; n, 4.

Since macrophages are the major PGE_2 producing cells in the spleen, we thought it would be of interest to determine the effects of thermal injury on PGE_2 production by these cells. Splenic macrophages are difficult to purify in the numbers required for these experiments, and therefore we used resident peritoneal macrophages instead. These cells were obtained by washing the peritoneal cavity with 10 ml of tissue culture medium; the cell suspensions contained $\geq$ 90% macrophages. Resident peritoneal macrophages were harvested from injured and sham-treated animals at 7 days postburn, and PGE_2 production by these cells was measured. Conditions were the same as those used with spleen cells, except the cells in lower numbers (2.5×10^6 cells/ml) were incubated for 24 h rather than 66 h for measurement of PGE_2 production. PGE_2 production by resident macrophages from injured animals was markedly increased as compared with sham-treated animals at all time points (Figure 5). Approximately tenfold more PGE_2 was produced by resident macrophages as compared with spleen cells (compare Figures 4 and 5). These results suggested that macrophages contributed to the increased PGE_2 production by spleen cells from injured animals.

To determine the involvement of PGE_2 in the depression of cell-mediated immune responses following thermal injury, we next determined the effects of NSAIDs on these responses. Because macrophages were found to be such effective producers of PGE_2, we first determined the effects of NSAID treatment on PGE_2 production by these cells. Resident macrophages from injured animals were incubated for 15 min at room temperature with 100 μM indomethacin as described in our studies with neutrophils [28]. A marked suppression of PGE_2 production by the indomethacin treatment was observed (Figure 6), indicating that cyclooxygenase was inhibited under the conditions of our experiments.

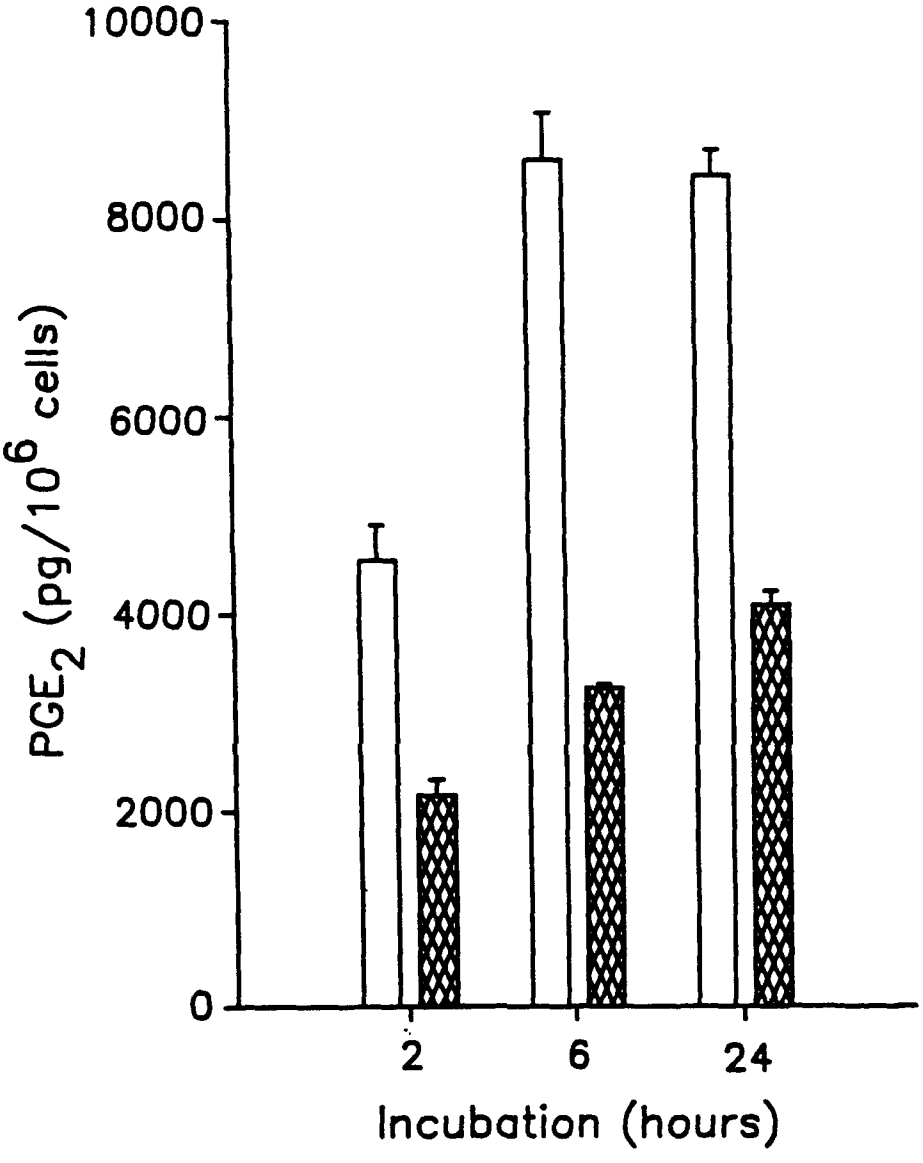

Figure 5. Autogenous PGE_2 production by resident peritoneal macrophages during short-term incubation in vitro. Resident peritoneal macrophages were harvested from injured animals (open bars) and sham-treated animals (cross-hatch bars) at seven days postburn. The macrophages were incubated for 24 h in vitro in the absence of activators, and PGE_2 in the supernatants was measured at various time intervals during the incubation period. Data are mean ± SEM; n, 4.

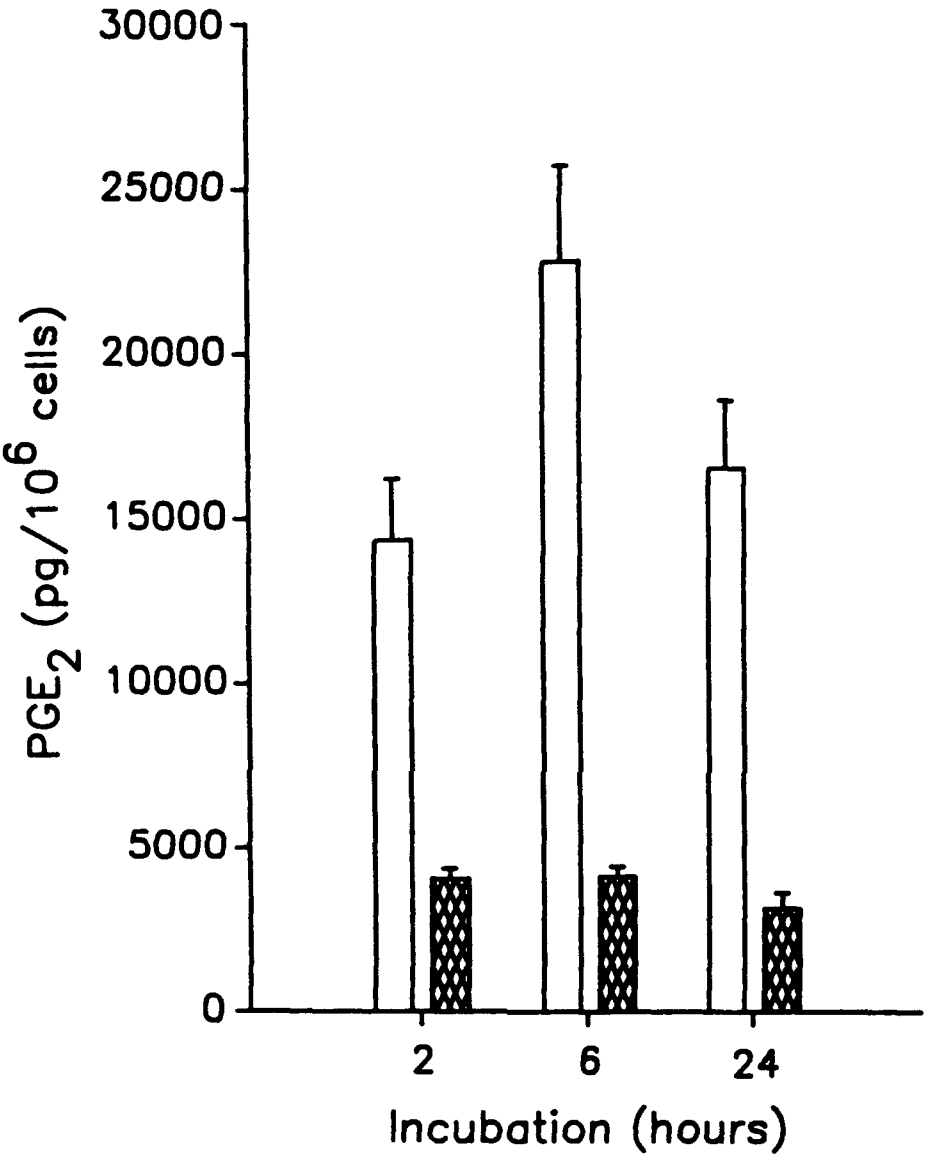

Figure 6. Inhibition of autogenous PGE_2 production by indomethacin. Resident peritoneal macrophages were harvested from injured animals at seven days postburn. The macrophages were treated with 100 μM indomethacin or received no treatment before incubation in vitro for 24 h in the absence of activators. PGE_2 in the supernatants was measured at various time intervals during the incubation period. Data are mean ± SEM; n, 4.

The effects of treatment with various NSAIDs on cell-mediated immune responses were next determined. Spleen cells were harvested from injured and sham-treated animals at 7 days postburn and treated with indomethacin, ibuprofen, piroxicam or buffer in vitro under the conditions described above. Proliferative responses to Con A and PHA and the primary immune response to sheep erythrocytes were then measured. None of the NSAIDs had any major effect on responses of spleen cells from injured or sham-treated animals (Tables 1 and 2). Since NSAID treatment suppressed PGE_2 production under the conditions of our experiments, these results suggested either that additional mediators were involved in depressing cell-mediated immune responses or that PGE_2-mediated suppressive effects were not readily reversible.

We next investigated the involvement of IFN_γ in the depression of cell-mediated immune responses following thermal injury. Proliferative responses of spleen cells to Con A and PHA and the primary immune response to sheep erythrocytes were measured in the presence and absence of 250 units of monoclonal antibody to IFN_γ (Amgen Biologicals, Thousand Oaks, CA). For these experiments, spleen cells were harvested from injured and sham-treated animals at 7 days postburn. Monoclonal antibody to IFN_γ had no major effect on responses of spleen cells from injured or sham-treated animals (Tables 3 and 4).

Since PGE_2 and IFN_γ act in concert to suppress lymphocyte function, we next determined the effects of monoclonal antibody to IFN_γ in combination with indomethacin on cell-mediated immune responses. Spleen cells were harvested from injured and sham-treated animals at 7, 10 and 14 days postburn, the times when cell-mediated immune responses were maximally depressed in the initial experiments. Spleen cells from injured animals were treated in vitro with 100 μM indomethacin alone. The cells were then

Table 1

Effect of NSAIDS on Proliferative Responses of Spleen Cells to Con A and PHA[a]

Source of Spleen Cells	Treatment	^{3}H-Thymidine Incorporation (cpm x 10^{-3}) Con A	PHA
Injured animals	Indomethacin	7.23 ± 1.22	3.97 ± 0.18
	Piroxicam	6.55 ± 0.79	3.52 ± 0.04
	Ibuprofen	9.25 ± 0.12	4.44 ± 0.45
	Buffer	7.35 ± 0.15	4.57 ± 0.35
	No treatment	9.05 ± 0.71	4.59 ± 0.09
Sham-treated animals	Indomethacin	28.64 ± 1.74	16.87 ± 0.31
	Piroxicam	22.23 ± 2.08	16.71 ± 0.52
	Ibuprofen	24.45 ± 2.15	17.78 ± 0.63
	Buffer	28.28 ± 2.75	17.36 ± 0.21
	No treatment	32.93 ± 0.61	19.33 ± 0.65

[a]Spleen cells harvested from injured and sham-treated animals at 7 days postburn were treated in vitro with NSAIDS or buffer before addition to the assays. Data are mean ± SEM; n, 3.

Table 2

Effect of NSAIDs on the In Vitro Primary Immune Response of Spleen Cells to Sheep Erythrocytes[a]

Source of Spleen Cells	Treatment	Plaque-Forming Cells (pfc x 10^{-3})
Injured animals	Indomethacin	0.93 ± 0.04
	Piroxicam	0.91 ± 0.14
	Ibuprofen	1.08 ± 0.20
	Buffer	1.02 ± 0.12
	No treatment	1.13 ± 0.12
Sham-treated animals	*Indomethacin*	3.30 ± 0.29
	Piroxicam	2.96 ± 0.31
	Ibuprofen	3.76 ± 0.40
	Buffer	3.58 ± 0.41
	No treatment	3.72 ± 0.24

[a]Spleen cells harvested from injured and sham-treated animals at 7 days postburn were treated in vitro with NSAIDs or buffer before addition to the assays. Data are mean ± SEM; n, 4.

Table 3

Effect of Monoclonal Antibody to IFN_γ on Proliferative Responses of Spleen Cells to Con A and PHA[a]

Source of Spleen Cells	Treatment	^{3}H-Thymidine Incorporation (cpm x 10^{-3}) Con A	PHA
Injured animals	Mab to IFN_γ	9.07 ± 0.43	16.71 ± 0.67
	No treatment	5.77 ± 0.65	14.74 ± 0.84
Sham-treated animals	Mab to IFN_γ	28.85 ± 1.53	34.27 ± 0.80
	No treatment	26.71 ± 0.89	36.99 ± 2.16

[a]Mab to IFN_γ (250 units) was added directly to the assays. Data are mean ± SEM; n, 3.

Table 4

Effect of Monoclonal Antibody to IFN_γ on the In Vitro Primary Immune Response to Sheep Erythrocytes[a]

Source of Spleen Cells	Treatment	Plaque-Forming Cells (pfc x 10^{-3})
Injured animals	Mab to IFN_γ	0.71 ± 0.10
	No treatment	0.57 ± 0.12
Sham-treated animals	Mab to IFN_γ	2.98 ± 0.37
	No treatment	2.54 ± 0.28

[a]Mab to IFN_γ (250 units) was added directly to the assays. Data are mean ± SEM; n, 4.

added to the assay together with 250 units of monoclonal antibody to IFN_γ. Indomethacin and monoclonal antibody to IFN_γ were tested alone in the controls. Proliferative responses of spleen cells from injured animals to Con A and PHA and the primary immune response to sheep erythrocytes were not affected by any of the treatments (Tables 5 and 6). These results do not support a role for IFN_γ in the depression of cell-mediated immune responses following thermal injury. If PGE_2 and IFN_γ are involved in the depression of these responses, then they must act early in the evolution of these alterations by non-reversible mechanisms.

Effects of Lipoxygenase Inhibitors on Arachidonic Acid Metabolism and on Cellular Alterations Induced by Thermal Injury

Since the studies described above failed to implicate cyclooxygenase metabolites in the depression of cell-mediated immune responses following thermal injury, we considered the possibility that lipoxygenase metabolites might be involved. To test this hypothesis, we selectively inhibited the lipoxygenase pathway by parenteral therapy with lipoxygenase inhibitors and determined effects on cell-mediated immune responses. For these experiments, we returned to the guinea pig model, since this is the superior model for measurement of arachidonate metabolites.

The lipoxygenase inhibitors employed in our experiments were diethylcarbamazine (Sigma Chemical Co., St. Louis, MO) and nafazatrom (Miles Pharmaceuticals, West Haven, CT). The drugs were administered intramuscularly at three hours postburn and then twice daily in doses of 7.5 mg/kg (total dose of 15 mg/kg/day) for the duration of the experiments. The placebo, 0.01 M phosphate buffered saline, pH 7.4, was administered twice daily in the same volume as the drugs.

We initially tested the selectivity of the lipoxygenase inhibitors

Table 5

Effect of Indomethacin and Monoclonal Antibody to IFN_γ on Proliferative Responses of Spleen Cells from Injured Animals to Con A and PHA[a]

Days Postburn	Treatment	^{3}H-Thymidine Incorporation (cpm x 10^{-3}) Con A	PHA
7	Mab to IFN_γ plus indomethacin	2.52 ± 0.09	2.24 ± 0.25
	Mab to IFN_γ alone	9.76 ± 0.25	8.96 ± 0.06
	Indomethacin alone	5.51 ± 0.51	4.24 ± 0.73
	No treatment	10.78 ± 0.78	8.95 ± 0.20
10	Mab to IFN_γ plus indomethacin	7.04 ± 0.20	7.72 ± 0.61
	Mab to IFN_γ alone	6.82 ± 0.49	7.74 ± 0.21
	Indomethacin alone	6.06 ± 0.50	5.75 ± 0.68
	No treatment	6.61 ± 0.59	7.46 ± 0.62
14	Mab to IFN_γ plus indomethacin	9.84 ± 0.58	9.01 ± 0.50
	Mab to IFN_γ alone	11.37 ± 0.88	12.96 ± 0.89
	Indomethacin alone	10.79 ± 0.91	9.44 ± 0.46
	No treatment	12.99 ± 1.35	10.57 ± 0.51

[a]Spleen cells harvested from injured animals at 7, 10 or 14 days postburn were treated in vitro with 100 μM indomethacin before addition to the assays. Mab to IFN_γ (250 units) was added directly to the assays. Responses of spleen cells from sham-treated animals ranged from 27.97 to 35.13 x 10^{-3} for Con A and 24.31 to 26.47 x 10^{-3} for PHA. Data are mean ± SEM; n, 3.

Table 6

Effect of Indomethacin and Monoclonal Antibody to IFN_γ on the In Vitro Primary Immune Response of Spleen Cells from Injured Animals to Sheep Erythrocytes[a]

Days Postburn	Treatment	Plaque-Forming Cells (pfc x 10^{-3})
7	Mab to IFN_γ plus indomethacin	0.75 ± 0.09
	Mab to IFN_γ alone	0.95 ± 0.15
	Indomethacin alone	0.81 ± 0.11
	No treatment	1.35 ± 0.18
10	Mab to IFN_γ plus indomethacin	1.17 ± 0.13
	Mab to IFN_γ alone	1.25 ± 0.10
	Indomethacin alone	1.10 ± 0.10
	No treatment	1.26 ± 0.17
14	Mab to IFN_γ plus indomethacin	1.15 ± 0.04
	Mab to IFN_γ alone	1.30 ± 0.04
	Indomethacin alone	1.23 ± 0.10
	No treatment	1.39 ± 0.22

[a]Spleen cells harvested from injured animals at 7, 10 or 14 days postburn were treated in vitro with 100 μM indomethacin before addition to the assays. Mab to IFN_γ (250 units) was added directly to the assays. Responses of spleen cells from sham-treated animals ranged from 3.10 to 3.34 x 10^{-3}. Data are mean ± SEM; n, 4.

under the conditions of our experiments. Injured animals were treated with the lipoxygenase inhibitors or placebo as described above, and metabolites of the lipoxygenase and cyclooxygenase pathways were measured in wound fluid at the site of thermal injury. Wound fluid was collected before injury and at various time intervals during four days postburn as previously described, except both 5 μM meclofenamate and 5 μM nordihydroguaiaretic acid were present during collection to inhibit artifactual generation of cyclooxygenase and lipoxygenase metabolites. Leukotriene (Lt) B_4, LtC_4, 6-keto-$PGF_{1\alpha}$ and thromboxane (Tx) B_2 were measured by radioimmunoassay using kits from Advanced Magnetics.

LtB_4 and LtC_4 began to increase in wound fluid from injured animals at two days postburn and reached maximal levels at four days postburn (Figures 7 and 8). The increase in 6-keto-$PGF_{1\alpha}$ and TxB_2 in wound fluid from the injured animals occurred earlier, at one day postburn, and was sustained for four days postburn (Figures 9 and 10). Considerable suppression of LtB_4 and LtC_4 production was observed during the four day postburn period in injured animals treated with diethylcarbamazine or nafazatrom (Figures 7 and 8). In contrast, production of 6-keto-$F_{1\alpha}$ and TxB_2 was slightly increased in these animals as compared with the placebo-treated animals (Figures 9 and 10). These results documented the selectivity of the lipoxygenase inhibitors and suggested that these inhibitors induced a partial shunting of arachidonic acid through the cyclooxygenase pathway.

The effects of parenteral therapy with lipoxygenase inhibitors on proliferative responses of spleen cells to T cell mitogens were next investigated. Proliferative responses to Con A and PHA were measured as previously described [14]. The lipoxygenase inhibitors had no effect on these responses in injured animals during nine days postburn (Figures 11 and 12). These results failed to implicate lipoxygenase metabolites in the depres-

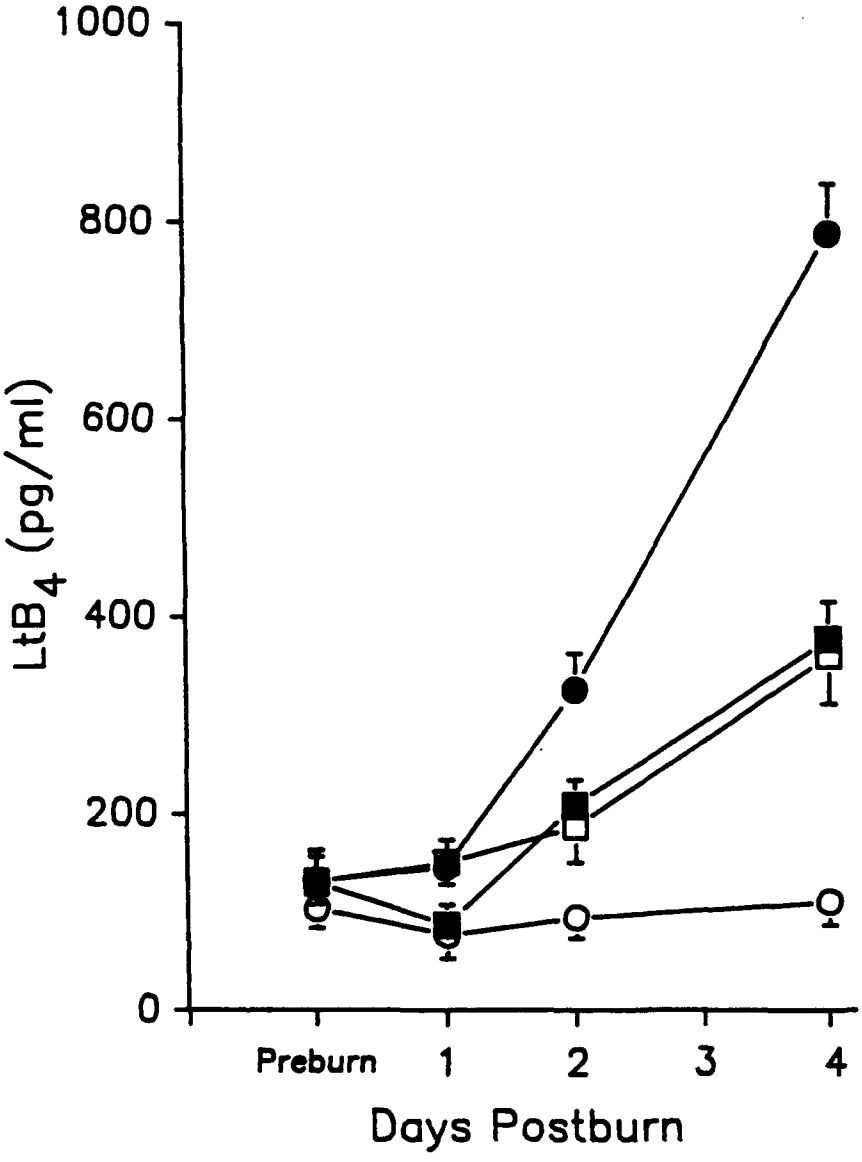

Figure 7. Effect of lipoxygenase inhibitors on LtB_4 concentrations in wound fluid during four days postburn. Injured animals were treated parenterally with diethylcarbamazine (open squares), nafazatrom (closed squares) or placebo (closed circles). Sham-treated animals (open circles) served as a control. There were five animals in each group. Data are mean ± SEM.

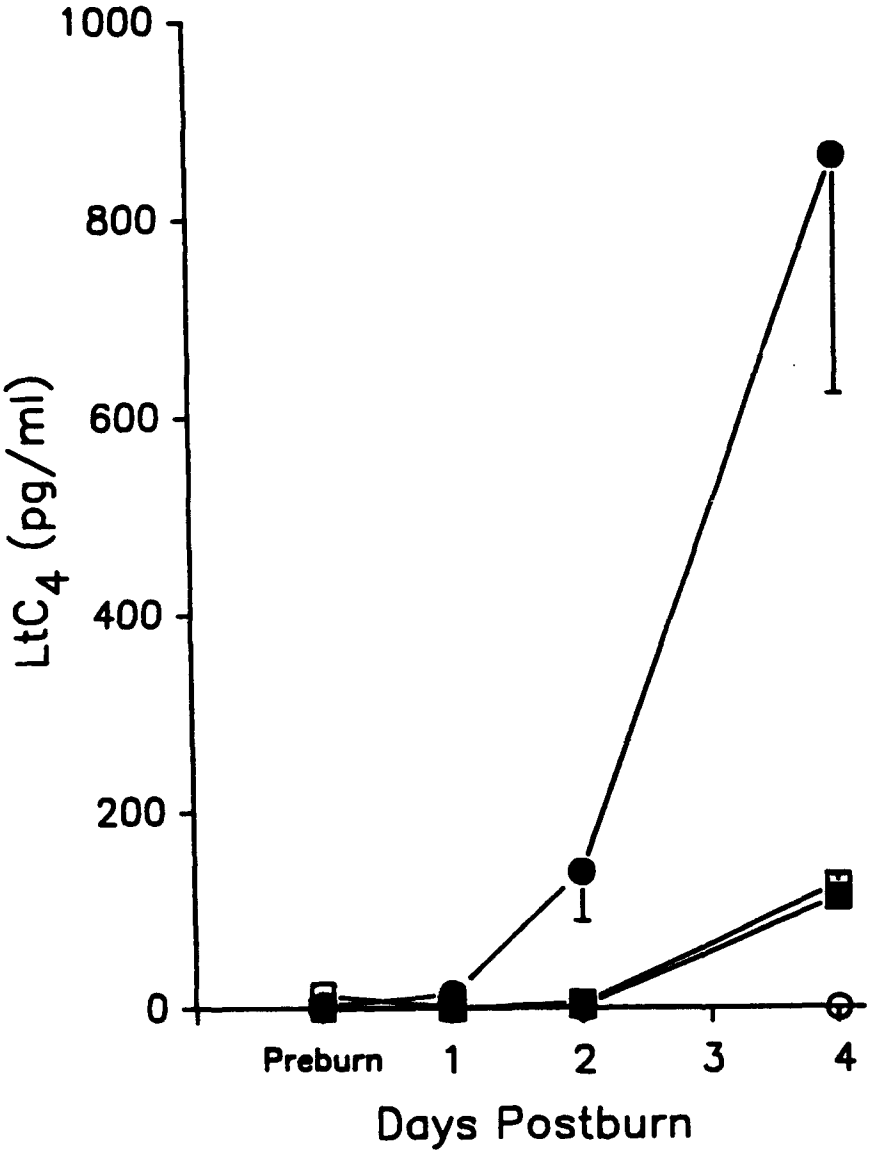

Figure 8. Effect of lipoxygenase inhibitors on LtC_4 concentrations in wound fluid during four days postburn. Injured animals were treated parenterally with diethylcarbamazine (open squares), nafazatrom (closed squares) or placebo (closed circles). Sham-treated animals (open circles) served as a control. There were five animals in each group. Data are mean ± SEM.

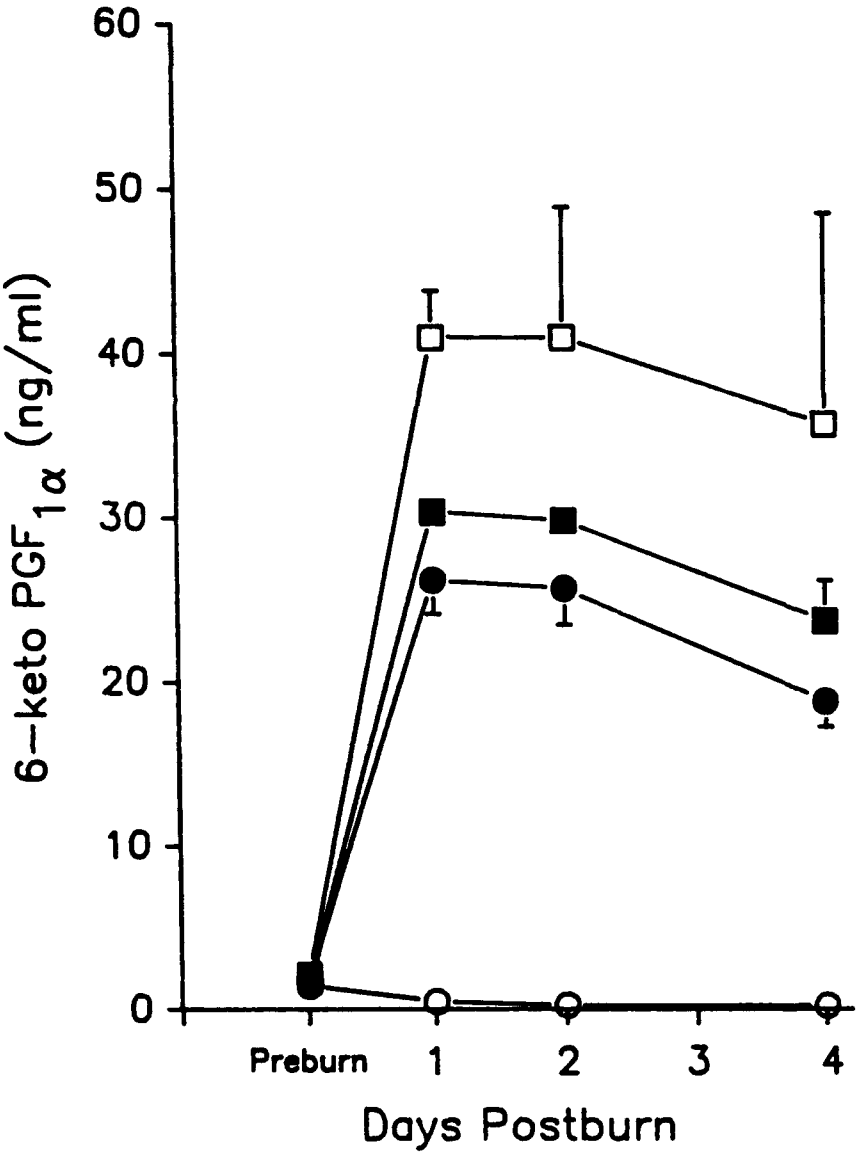

Figure 9. Effect of lipoxygenase inhibitors on 6-keto-$PGF_{1\alpha}$ concentrations in wound fluid during four days postburn. Injured animals were treated parenterally with diethylcarbamazine (open squares), nafazatrom (closed squares) or placebo (closed circles). Sham-treated animals (open circles) served as a control. There were five animals in each group. Data are mean ± SEM.

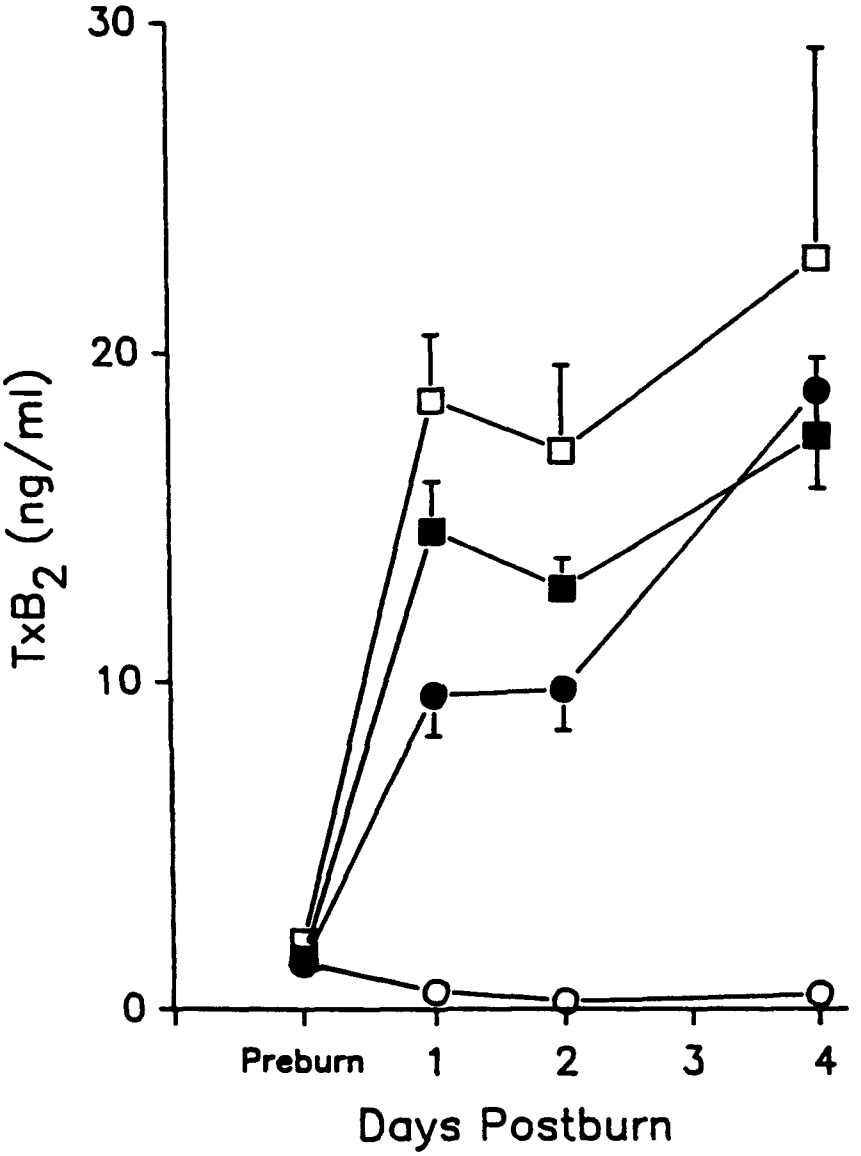

Figure 10. Effect of lipoxygenase inhibitors on TxB_2 concentrations in wound fluid during four days postburn. Injured animals were treated parenterally with diethylcarbamazine (open squares), nafazatrom (closed squares) or placebo (closed circles). Sham-treated animals (open circles) served as a control. There were five animals in each group. Data are mean ± SEM.

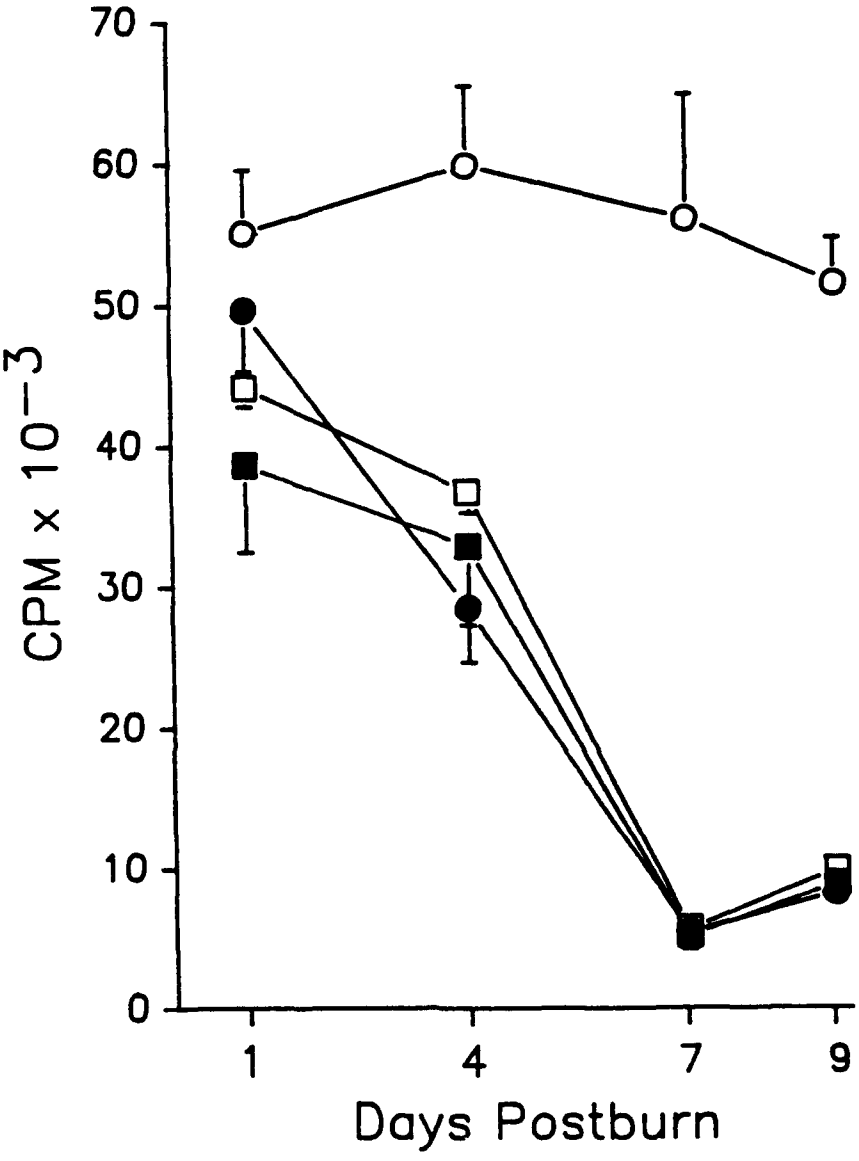

Figure 11. Effect of lipoxygenase inhibitors on proliferative responses of spleen cells to Con A during nine days postburn. Injured animals were treated parenterally with diethylcarbamazine (open squares), nafazatrom (closed squares) or placebo (closed circles). Sham-treated animals (open circles) served as a control. There were three animals in each group. Data are mean ± SEM.

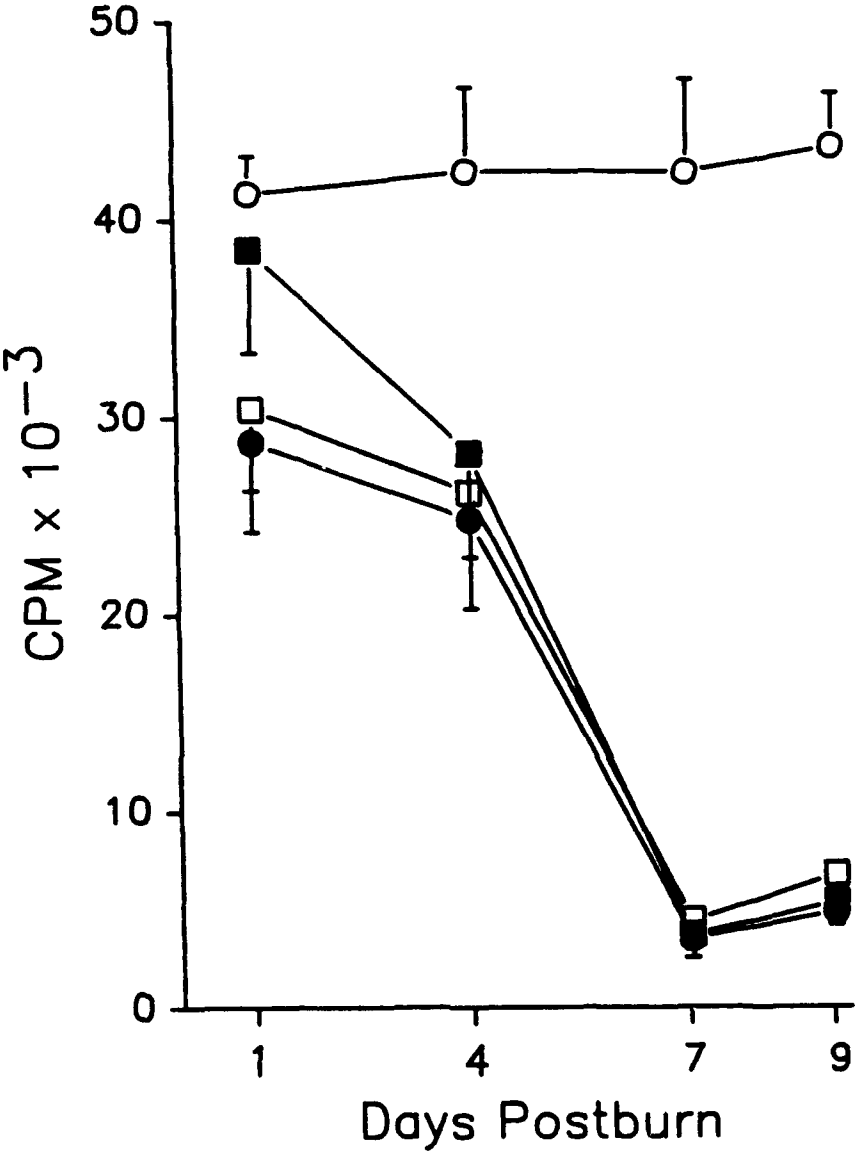

Figure 12. Effect of lipoxygenase inhibitors on proliferative responses of spleen cells to PHA during nine days postburn. Injured animals were treated parenterally with diethylcarbamazine (open squares), nafazatrom (closed squares) or placebo (closed circles). Sham-treated animals (open circles) served as a control. There were three animals in each group. Data are mean ± SEM.

sion of lymphoproliferative responses to mitogens following thermal injury.

We also tested the effects of parenteral therapy with the lipoxygenase inhibitors on the bactericidal activity of neutrophils in the injured animals. Bactericidal activity against *P. aeruginosa* was measured as previously described [28]. The lipoxygenase inhibitors had no effect on bactericidal activity of neutrophils in injured animals during seven days postburn (Figure 13). These results serve as a good control for our experiments employing cyclooxygenase inhibitors in which correction of the bactericidal defect of neutrophils was demonstrated with these drugs (Refer to the section on effects of cyclooxygenase inhibitors on immunologic alterations).

Major Conclusions

We characterized the mechanism responsible for the bactericidal defect of neutrophils in a guinea pig model of thermal injury. This defect was found to be related to elevation of intracellular cAMP, which in turn was related to autogenous PGE_1 production by the neutrophils. E type prostaglandins and other adenylate cyclase agonists in body fluids also may contribute to the elevation of intracellular cAMP in neutrophils.

We also demonstrated that the bactericidal defect of neutrophils induced by thermal injury in the guinea pig model was reversible, being fully amenable to correction in vitro or in vivo with NSAIDs. These drugs acted by reducing intracellular cAMP through cyclooxygenase inhibition and possibly also by additional mechanisms.

We did not confirm the hypothesis that cyclooxygenase or lipoxygenase metabolites of arachidonic acid metabolism or IFN_γ play a major role in mediating the depression of cell-mediated immune responses following thermal injury in guinea pig or mouse models. If these compounds are involved

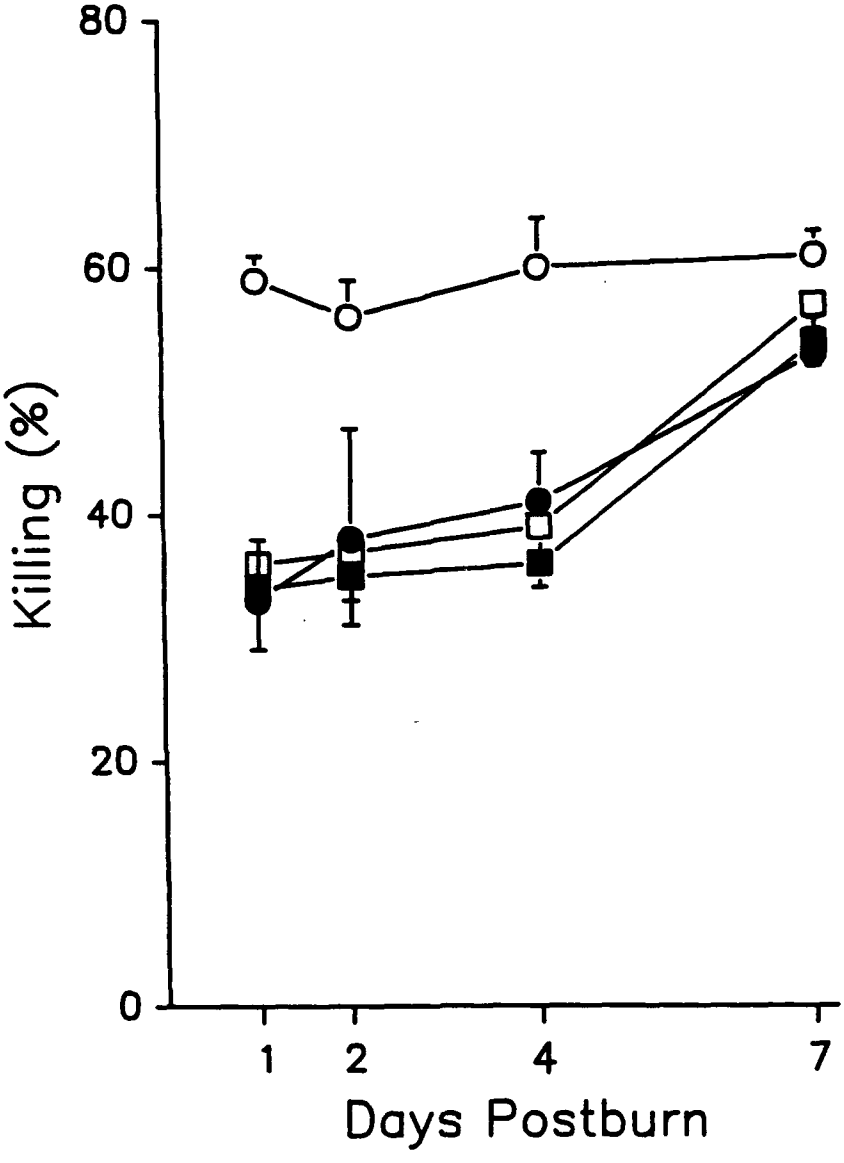

Figure 13. Effect of lipoxygenase inhibitors on bactericidal activity of neutrophils against *P. aeruginosa* during seven days postburn. Injured animals were treated parenterally with diethylcarbamazine (open squares), nafazatrom (closed squares) or placebo (closed circles). Sham-treated animals (open circles) served as a control. Neutrophils from two to three animals in each group were separately pooled and then assayed. Data are mean ± SEM; n, 2.

in depressing cell-mediated immune responses following thermal injury, then their effects are not readily reversible. Other mechanisms may be primarily responsible for these alterations.

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PERSONNEL RECEIVING CONTRACT SUPPORT, GRADUATE DEGREES

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Graduate Degrees: None